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Maternal phthalate exposure promotes allergic airway inflammation over 2 generations through epigenetic modifications



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Background: Prenatal and early postnatal exposures to environmental factors are considered responsible for the increasing prevalence of allergic diseases. Although there is some evidence for allergy-promoting effects in children because of exposure to plasticizers, such as phthalates, findings of previous studies are inconsistent and lack mechanistic information.

Objective: We investigated the effect of maternal phthalate exposure on asthma development in subsequent generations and their underlying mechanisms, including epigenetic alterations.

Methods: Phthalate metabolites were measured within the prospective mother-child cohort Lifestyle and Environmental Factors and Their Influence on Newborns Allergy Risk (LINA) and correlated with asthma development in the children.

A murine transgenerational asthma model was used to identify involved pathways.

Results: In LINA maternal urinary concentrations of mono-n-butyl phthalate, a metabolite of butyl benzyl phthalate (BBP),

were associated with an increased asthma risk in the children. Using a murine transgenerational asthma model, we demonstrate a direct effect of BBP on asthma severity in the offspring with a persistently increased airway inflammation up to the F2 generation. This disease-promoting effect was mediated by BBP-induced global DNA hypermethylation in CD4⁺ T cells of the offspring because treatment with a DNA-demethylating agent alleviated exacerbation of allergic airway inflammation. Thirteen transcriptionally downregulated genes linked to promoter or enhancer hypermethylation were identified. Among these, the GATA-3 repressor zinc finger protein 1 (*Zfpml*) emerged as a potential mediator of the enhanced susceptibility for T_H2-driven allergic asthma. **Conclusion:** These data provide strong evidence that maternal BBP exposure increases the risk for allergic airway inflammation in the offspring by modulating the expression of genes involved in T_H2 differentiation through epigenetic alterations. (J Allergy Clin Immunol 2018;141:741-53.)

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Key words: Airway inflammation, asthma, phthalates, epigenetics, T cells

Allergic asthma is caused by a T_H2 cell-mediated immune response to common environmental allergens and characterized by airway inflammation with pulmonary eosinophilia, airway hyperreactivity (AHR), and increased serum IgE levels.¹ This inflammatory airway disease has a strong genetic background, but also, a multitude of environmental trigger factors have been described.^{2,3} Epidemiologic and experimental studies demonstrated that exposure to environmental pollutants, such as diesel exhaust particles or environmental tobacco smoke, or chemicals, such as volatile organic compounds, can lead to exacerbation of respiratory symptoms and asthma.⁴⁻⁷ In particular, the prenatal and early postnatal periods appear critical to environmental exposures, probably interfering with the developmental programming of the immune system and thereby altering the disease risk in later life.⁸ Epigenetic changes, such as modified DNA methylation, have been described as mediators between exposure and disease development.^{8,9} This has been shown for allergy-preventing effects induced by upbringing in a farming environment or exposure to microbial components, as well as for asthma-promoting influences, such as maternal exposure to tobacco smoke during pregnancy.⁹⁻¹¹ These findings emphasize that the developing immune system is especially susceptible to perturbations by external factors.

Here, we focused on phthalates, a group of chemicals commonly used as plasticizers in large quantities worldwide. These chemicals are present in a wide range of consumer products, such as cosmetics, plastics, floor coverings, building materials, toys, and cleaning products.^{12,13} Human subjects are exposed to phthalates mainly through ingestion or inhalation throughout life, starting *in utero*.^{14,15} Although there is some evidence of allergy-promoting effects in children by maternal phthalate exposure, findings of previous studies are conflicting regarding the effect direction, the investigated exposure conditions, and their assessment.¹⁶⁻¹⁸ Furthermore, little is known about the mechanisms of maternal phthalate exposure, leading to an altered disease risk in children's later life.

In the present study we report a significant association between maternal urinary concentrations of the phthalate metabolite mono-n-butyl phthalate (MnBP) and an increased asthma risk in children of our prospective mother-child cohort Lifestyle and Environmental Factors and Their Influence on Newborns Allergy Risk (LINA).⁷ Using a murine transgenerational asthma model, we demonstrate that maternal exposure to butyl benzyl phthalate (BBP), a parent compound of MnBP, has a direct effect on severity of allergic airway inflammation in the offspring, observing an enhanced airway inflammation, even in the F2 generation. This asthma-promoting effect was mediated by BBP-induced DNA hypermethylation, as shown by diminished BBP effects after treating mice with a hypomethylation-inducing compound and a subsequently reduced expression of genes involved in the differentiation of T_H2 cells, such as the GATA-3 repressor zinc finger protein 1 (*Zfpml*), offering an explanation for the increased allergic immune response.

METHODS

For more detailed information, please see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Abbreviations used

AHR:	Airway hyperreactivity
Aza:	5-Aza-2'-deoxycytidine
BBP:	Butyl benzyl phthalate
DEG:	Differentially expressed gene
DMR:	Differentially methylated region
Fads1:	Fatty acid desaturase 1
Fanca:	Fanconi anemia complementation group A
Foxp3:	Forkhead box p3
LINA:	Lifestyle and Environmental Factors and Their Influence on Newborns Allergy Risk
MBzP:	Monobenzyl phthalate
MnBP:	Mono-n-butyl phthalate
OVA:	Ovalbumin
WGBS:	Whole-genome bisulfite sequencing
Zfpml:	Zinc finger protein 1

LINA study design and sample collection

The LINA cohort study recruited 629 mother-child pairs between May 2006 and December 2008 in Leipzig, Germany, to investigate how environmental factors in the prenatal and postnatal period influence immune system development and the resulting disease risks.¹⁹⁻²¹

Urine samples and sample preparation

Phthalate metabolite quantification was carried out for 540 early morning maternal urine samples, as described previously.²²

Exposure to BBP and ovalbumin immunization

BALB/c mice were exposed to BBP (3 µg/mL) exclusively through drinking water (custom-built glass bottles) for different time periods, as described in detail in the [Methods](#) section in this article's Online Repository.

Measurement of airway responsiveness

Lung resistance was measured by using invasive plethysmography in response to inhaled methacholine, as described previously²³⁻²⁵ and in more detail in the [Methods](#) section in this article's Online Repository.

Whole-genome bisulfite sequencing, differentially methylated region calling, and annotation

Whole-genome bisulfite sequencing (WGBS) library construction and sequencing was performed, as previously described,²⁶ by applying a tagmentation-based, ultralow-input library preparation protocol with a quantity of 20 ng of input DNA. Sequencing and alignment (mm10) were carried out, as described previously.²⁷ Differentially methylated region (DMR) calling was performed, as described earlier.²⁷

RNA sequencing

Total RNA was isolated, and RNA sequencing was performed, as described previously.²⁸⁻³⁰

Mapping of DMRs to target genes

DMRs were mapped on gene bodies, promoters, and putative enhancers of the differentially expressed genes (DEGs), as described in detail in the [Methods](#) section in this article's Online Repository.

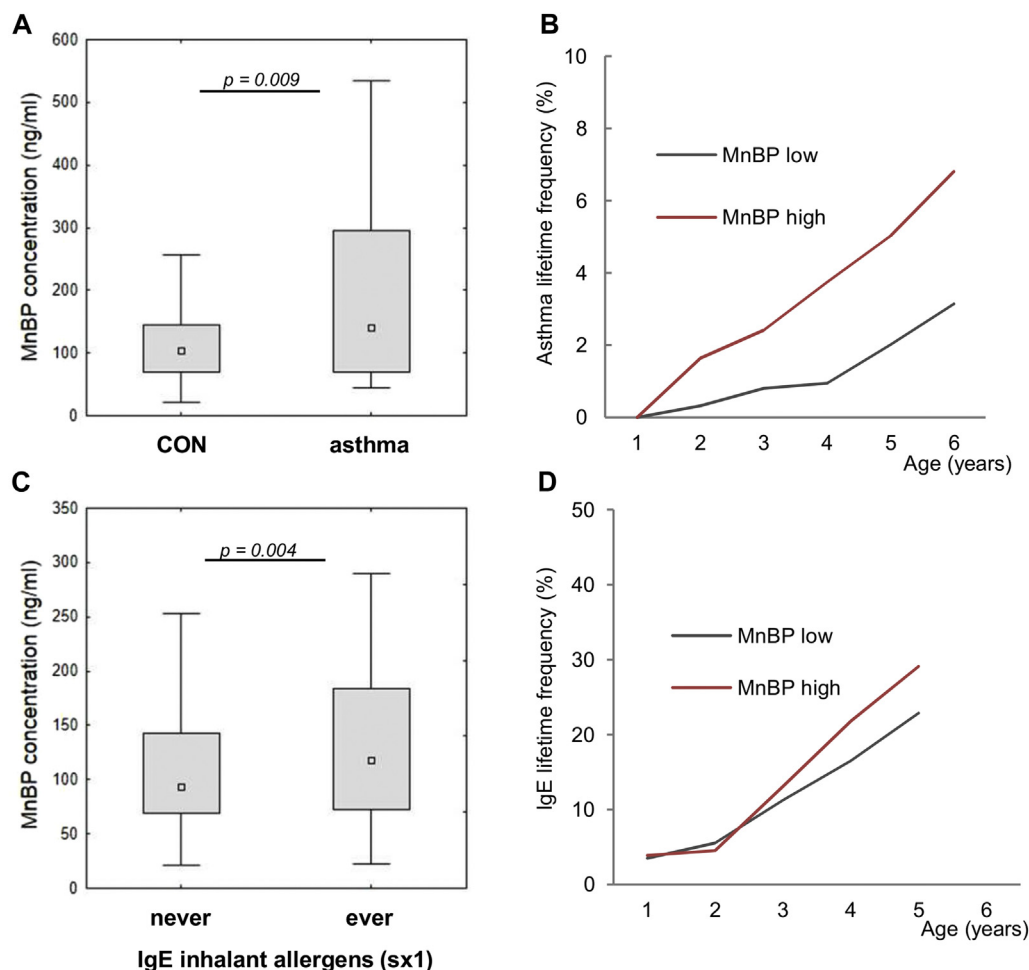


FIG 1. Maternal phthalate exposure and children's asthma and allergic sensitization. **A** and **C**, Maternal urine MnBP concentrations (34th gestational week) for children with asthma until age 6 (Fig 1, A) and sensitization against inhalant allergens (Fig 1, C) compared with control subjects (medians and interquartile ranges). **B** and **D**, Frequency and risk (adjusted odds ratio per 100 ng/mg MnBP increase) for asthma (Fig 1, B) and sensitization against inhalant allergens (Fig 1, D) according to maternal MnBP concentrations (high = greater than median).

MassARRAY methylation analysis

MassARRAY analysis was performed, as described previously.²⁷

Statistical analysis

Equal parameter distribution was tested by using χ^2 tests. Logistic regression models were implemented, adjusting for known confounding factors of lung disease or atopy in early childhood, to assess the contribution of phthalate exposure on risk increase for asthma development or increased IgE levels in the children.

Nonparametric tests were applied for all nonnormally distributed parameters (eg, Mann-Whitney *U* test [MWU] and Spearman correlations). All calculations were performed in STATISTICA software for Windows (Version 10; Statsoft, Tulsa, Okla). Data were expressed as means \pm SEMs, and *P* values of less than .05 were considered significant.

RESULTS

Association between maternal phthalate metabolites and the development of allergic asthma in children (LINA)

For the LINA mother-child study, 629 mother-child pairs were recruited between 2006 and 2008. The 6-year follow-up investigation included 420 (66.8%) of the families. Regarding asthma

development, only physician-diagnosed asthma was considered for this study. For 24 of the LINA children, an asthma diagnosis was reported until the age of 6 years. General characteristics of the study participants are shown in Table E1 in this article's Online Repository at www.jacionline.org. Apart from a slightly lower number of tobacco smoke-exposed children participating until the age of 6 years, there were no differences between the analyzed subcohort (*n* = 371) and the total LINA cohort (*n* = 629).

The concentration of 10 phthalate metabolites was determined in a total of 540 maternal urine samples of the 34th week of gestation. However, only measurements of the 371 children who participated in the study until age 6 were considered for our analysis (see Table E2 in this article's Online Repository at www.jacionline.org). Interestingly, an increased risk of asthma symptoms until age 6 was observed only in children from mothers with increased urinary MnBP levels (Fig 1, A and B), whereas all other metabolites showed no significant association (see Table E3 and E5 in this article's Online Repository at www.jacionline.org). In addition to the lung phenotype, high maternal MnBP concentrations were also associated with a higher risk for allergic sensitization against inhalant allergens in the children

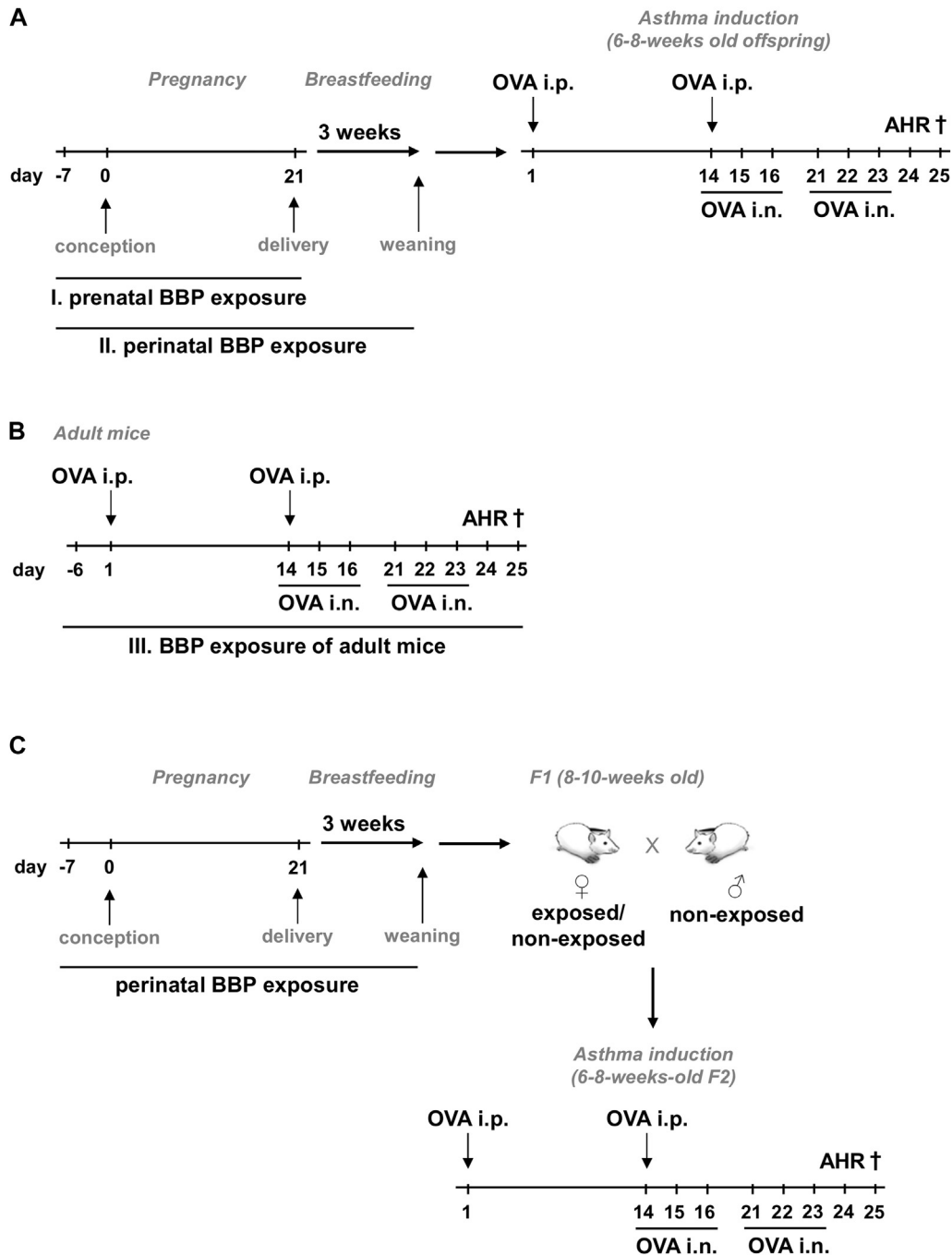


FIG 2. Phthalate exposure and induction of an asthma-like phenotype. **A**, Dams were exposed to BBP during pregnancy (prenatal) or until weaning when pups were 3 weeks old (perinatal exposure). **B**, Exposure of adult mice to BBP started 1 week before the first allergen injection and was continued until the end of the asthma induction protocol. **C**, Female F1 mice from exposed or control dams were mated with unexposed male mice. *i.n.*, Intranasal; *i.p.*, intraperitoneal.

(Fig 1, C and D, and see Table E4 and E5 in this article's Online Repository at www.jacionline.org).

Prenatal and perinatal exposure to BBP increased allergic airway inflammation in a murine asthma model

Next to monobenzyl phthalate (MBzP), MnBP is the major metabolite of BBP.³¹ To further investigate whether the observed

increase in asthma risk in the human study cohort might be directly caused by maternal exposure to BBP, we used a well-established transgenerational ovalbumin (OVA)-induced asthma mouse model.^{10,23,32,33} During the exposure period, BALB/c mice received 3 μ g/mL BBP through drinking water, a concentration relevant for human exposure. MnBP levels measured in the urine of BBP-exposed dams (see Table E6 in this article's Online Repository at www.jacionline.org) were comparable with those detected in highly exposed LINA mothers

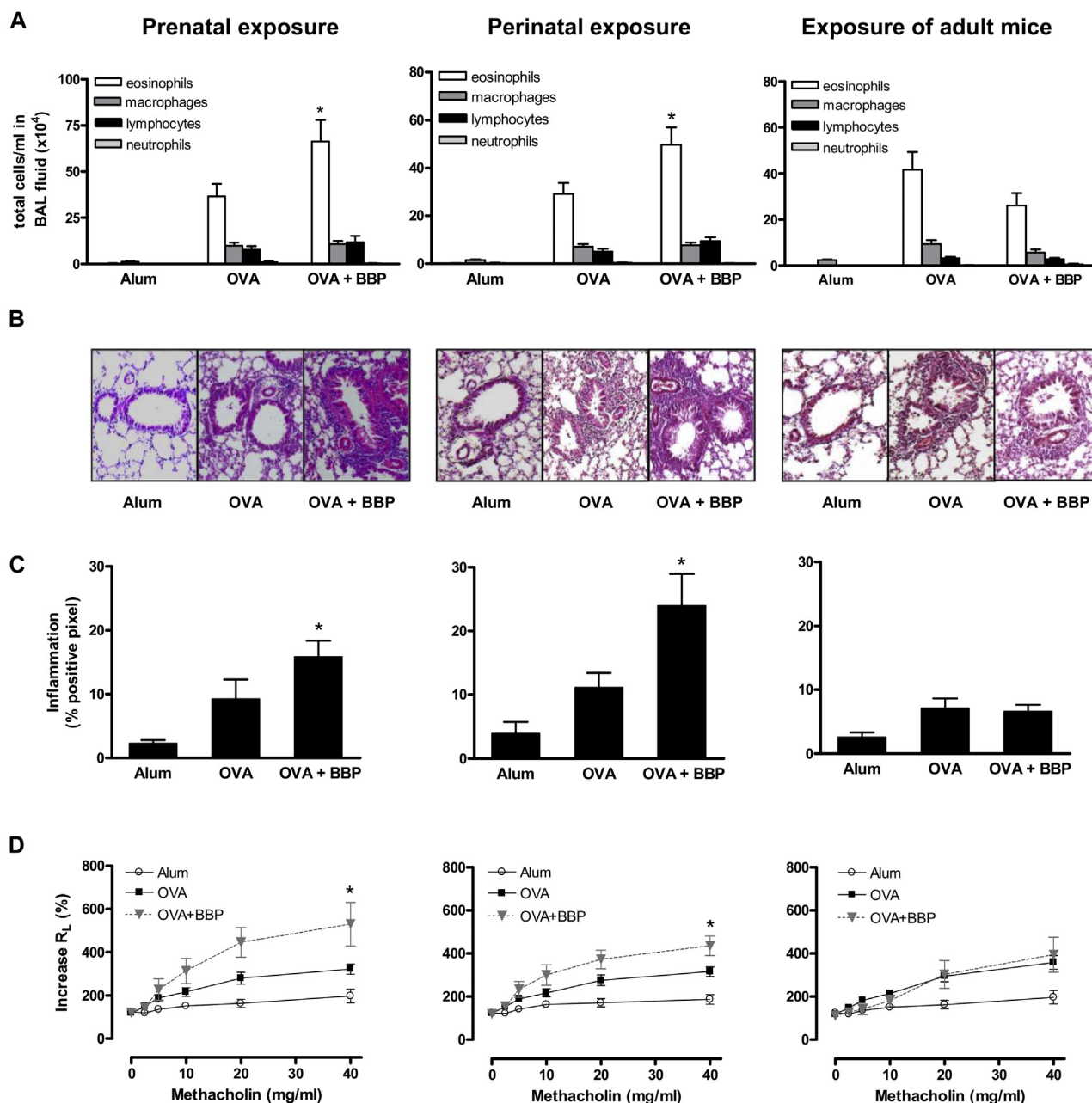


FIG 3. Prenatal and perinatal exposure to BBP increases allergic airway inflammation in a murine asthma model. Total cell numbers in bronchoalveolar lavage (BAL) fluid (**A**), airway inflammation examined by lung histology (hematoxylin and eosin, magnification $\times 100$; **B**) quantified by an investigator-independent computer-based analysis (**C**), and lung resistance (**D**) were examined in offspring from BBP-exposed dams and in BBP-exposed adult mice. Data are expressed as means \pm SEMs ($n \geq 9$ animals per group in the offspring [from ≥ 5 dams] and adult mice from 4 independent experiments). * $P < .05$.

(12.47–6894.87 ng/mL in maternal urine). Dams were exposed to BBP during pregnancy (prenatal) or during pregnancy and breast-feeding (perinatal) to analyze the effect of maternal BBP exposure on allergic airway inflammation. Grown-up offspring were then subjected to antigen sensitization without being exposed further to BBP (Fig 2, A). In another treatment regimen, adult mice were exposed to BBP starting 1 week before OVA sensitization until the end of the asthma protocol (Fig 2, B).

Although exposure of adult mice to BBP had no effect on the asthma-like phenotype, prenatal and perinatal BBP exposure significantly increased the number of eosinophils within the bronchoalveolar lavage fluid compared with the offspring from unexposed dams (Fig 3, A). Maternal BBP exposure led to substantially more inflammatory infiltrates in the airways, as demonstrated by means of histologic examination of hematoxylin and eosin–stained lung sections (Fig 3, B), as confirmed by means of software-based image analysis (Fig 3, C). Accordingly, AHR

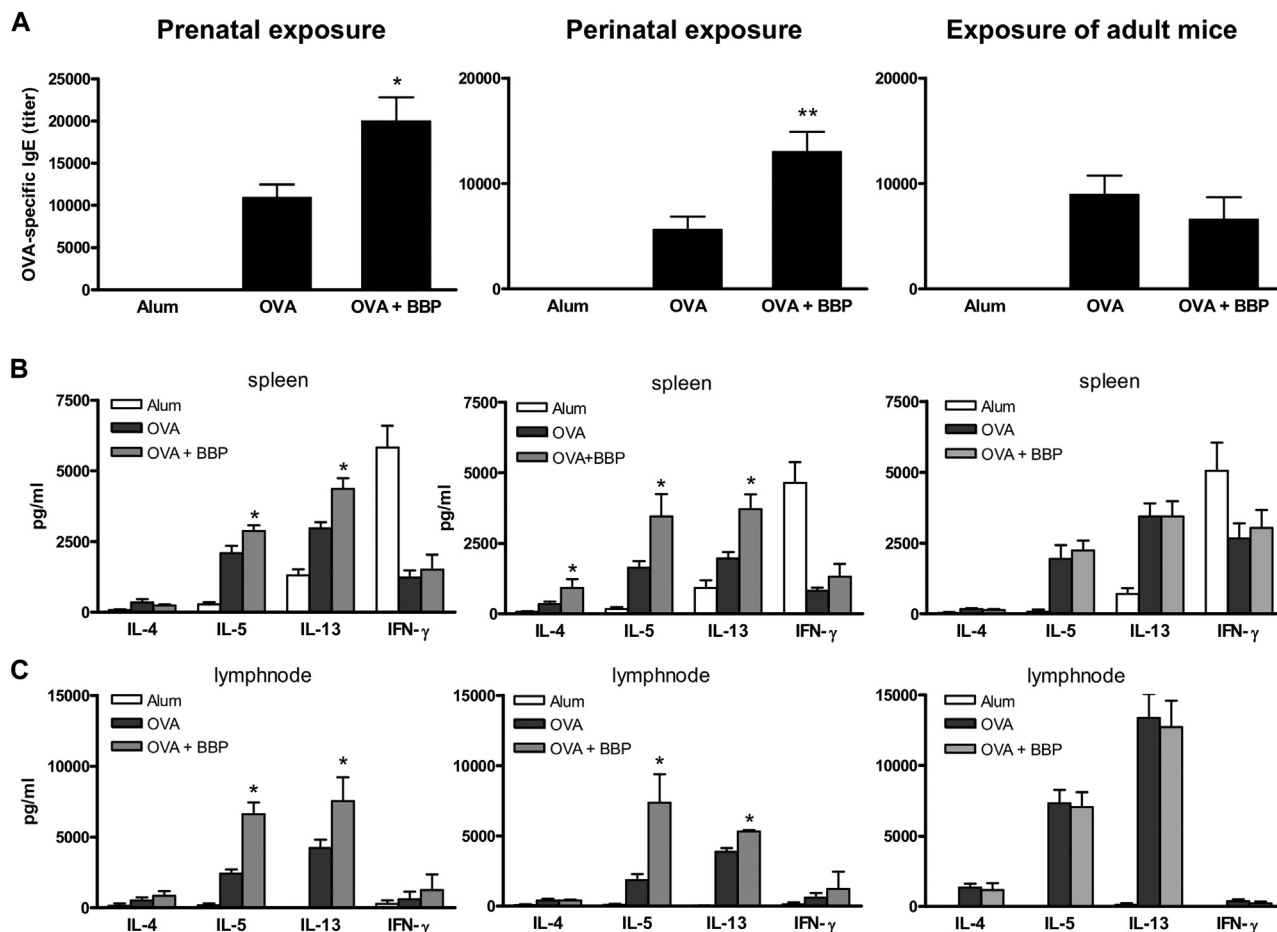


FIG 4. Prenatal and perinatal exposure to BBP enhances allergen-specific IgE levels and T_H2 cytokine production. OVA-specific IgE serum levels (**A**) and cytokine production of restimulated splenocytes (**B**) and lymph node cells (**C**) were analyzed in OVA-immunized offspring from BBP-exposed dams and in BBP-exposed adult mice. Data are expressed as means \pm SEMs ($n \geq 9$ animals per group in the offspring [from ≥ 5 dams] and adult groups from 4 independent experiments). * $P < .05$ and ** $P < .01$.

measured as lung resistance was enhanced in the offspring from BBP-exposed dams (Fig 3, D).

Furthermore, OVA-specific IgE levels were increased in prenatally and perinatally exposed offspring compared with control mice (Fig 4, A). Moreover, OVA-restimulated spleen and lymph node cells from offspring of BBP-exposed dams produced significantly higher amounts of the T_H2 cytokines IL-5 and IL-13, whereas there was no significant effect on the T_H1 cytokine IFN- γ (Fig 4, B and C).

To evaluate which BBP metabolite promotes the occurrence of an asthma-like phenotype, we perinatally exposed mice to MnBP or MBzP and subsequently characterized the asthma phenotype in the offspring. Interestingly, neither MnBP nor MBzP significantly increased the allergic airway inflammation (see Fig E1 in this article's Online Repository at www.jacionline.org).

Maternal BBP exposure induces global hypermethylation mediating the asthma-promoting effect

Early environmental influences have been shown to perturb epigenetic changes, such as DNA methylation, that might

contribute to programming of disease risks.⁸ To evaluate whether maternal (perinatal) BBP exposure alters the global DNA methylation pattern in the F1 generation, we subjected splenic $CD4^+$ T cells isolated from dams and their 3-week-old offspring to WGBS. DMRs were called with a difference in the methylation level of greater than 5% and a P value of less than .05 between the exposed group (F0 or F1) versus nonexposed control mice of the same generation ($n = 3$, median false discovery rate: F0, 0.056; F1, 0.036).

Our data show that maternal BBP exposure induced global DNA hypermethylation in the F0 generation (63% hypermethylated regions among identified DMRs) and to an even higher extent in the offspring (83%; Fig 5, A, data set 1). Similar to the F0 generation, hypermethylation in the F1 generation was not restricted to any particular genomic region but occurred genome wide (Fig 5, B).

One-week-old pups from BBP-exposed dams were treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (Aza) for 2 weeks until weaning to evaluate whether BBP-induced hypermethylation is linked to the severe allergic airway inflammation observed in the offspring.³⁴ Treatment of the offspring with Aza reversed enhanced airway inflammation caused by maternal BBP exposure, as demonstrated by the

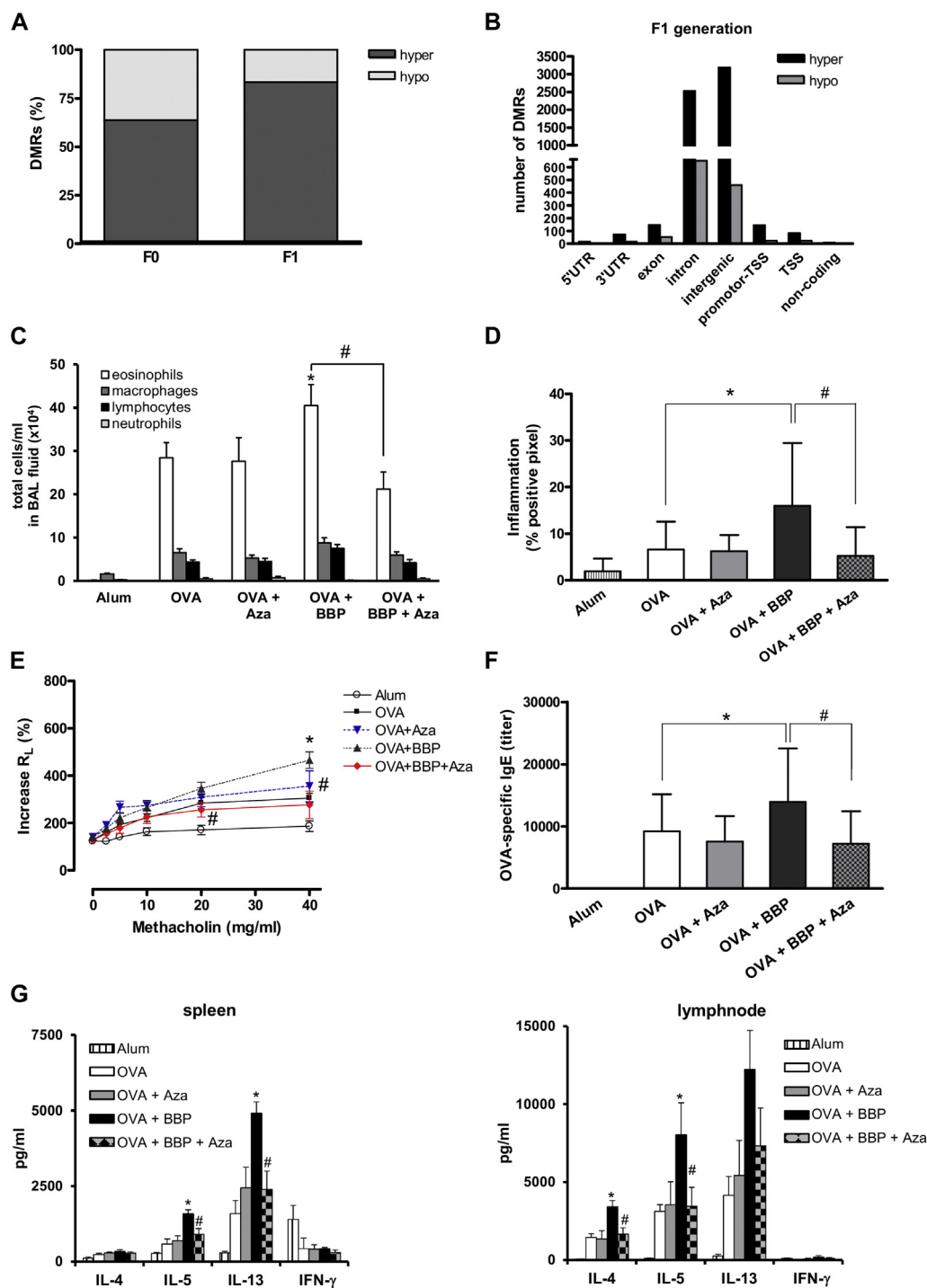


FIG 5. Maternal BBP exposure induces global hypermethylation, which mediates the asthma-promoting effect. **A**, DMRs were called from dams and their offspring between the exposed (F0 or F1) and nonexposed groups. **B**, Numbers of DMRs in different DNA regions of the F1 generation. **C–G**, Total cell numbers in bronchoalveolar lavage (BAL) fluid (Fig 5, C), airway inflammation (Fig 5, D), lung resistance (Fig 5, E), OVA-specific IgE levels (Fig 5, F), and cytokine production in spleen and lymph node cells (Fig 5, G) were examined in offspring from BBP-exposed dams compared with F1 mice treated with the DNA methyltransferase inhibitor Aza. Data are expressed as means \pm SEMs ($n \geq 9$ animals per group in the offspring [from ≥ 6 dams] from 6 independent experiments). * $P < .05$, OVA versus OVA + BBP. # $P < .05$, OVA + BBP versus OVA + BBP + Aza.

reduced number of eosinophilic granulocytes in bronchoalveolar lavage fluid (Fig 5, C), diminished lung inflammation (Fig 5, D), and attenuated OVA-specific lung resistance (Fig 5, E), reduced IgE levels (Fig 5, F), and IL-5/IL-13 levels in splenocytes and IL-4/IL-5 levels in lymph node cells (Fig 5, G). Aza treatment of pups from unexposed dams had no effect on the asthma-like phenotype in the offspring.

BBP-induced hypermethylation leads to downregulation of certain target genes

To characterize the functional consequences of BBP-induced hypermethylation on transcription, we assessed gene expression using RNA sequencing in CD4⁺ T cells from 3-week-old offspring alongside with our WGBS analysis. At a false discovery rate of less than 0.01, we detected 1138 differential expressed genes (DEGs) in the offspring of BBP-exposed dams in comparison with those of nonexposed mice. Treated versus nontreated mice clustered with respect to their genome-wide RNA expression by means of unsupervised clustering (Fig 6, A, data set 2). Two hundred forty-two of the obtained DEGs could be linked to 346 DMRs (Fig 6, B). Those target genes most likely mediating the BBP-related asthma promotion should fulfill certain criteria (see Fig E2 in this article's Online Repository at www.jacionline.org and data set 3). As already mentioned, BBP-induced hypermethylation was correlated with increased airway inflammation, which was alleviated by treatment with the DNA-demethylating agent Aza. Therefore, in a first step, we concentrated on those DEGs related to hypermethylated DMRs (201 DEGs) located in functionally translating regulatory elements (promoter/enhancer; 33 DEGs remaining). Because DNA hypermethylation is a key mechanism to gene silencing, we focused only on those transcriptionally repressed genes, leaving us with 13 potential candidates (data set 3). Among those, 3 genes were previously described to play a role in T_H2-driven allergic immune diseases and were subsequently evaluated in more detail.

Fatty acid desaturase 1 (*Fads1*) is an enzyme that regulates desaturation of fatty acids and is involved in the synthesis of long-chain polyunsaturated fatty acid. It has been shown that downregulation of the *Fads* gene is associated with lower polyunsaturated fatty acid levels and atopic eczema.³⁵ The second gene, Fanconi anemia complementation group A (*Fanca*), regulates the activity of regulatory T cells. Deletion of *Fanca* leads to a decreased efficiency to suppress effector T-cell function.³⁶ Third, we focused on *Zfpml*, also known as friend of GATA protein 1 (*Fog1*), a gene that acts as a repressor of GATA-3-mediated T_H2 cell development.³⁷⁻³⁹ Therefore downregulation of *Zfpml* could promote a T_H2-driven immune response. Validation of our RNA-sequencing results by using real-time PCR confirmed a reduced expression of all 3 genes in CD4⁺ T cells from 3-week-old offspring of BBP-exposed dams compared with control animals (Fig 6, C). In contrast, in BBP-exposed F0 mice expression of *Fads1*, *Fanca*, and *Zfpml* was not altered (Fig 6, C). Furthermore, downregulation of *Fanca* and *Zfpml* was also seen in full-grown offspring of BBP-exposed dams (8 to 12 weeks old), whereas the effect of maternal BBP exposure on *Fads1* expression in adult mice was not significant (Fig 6, C). Aza treatment of BBP-treated pups not only alleviated the asthma-promoting effect, as described earlier, but also abrogated downregulation of *Fanca* and *Zfpml* in the mature F1 generation (Fig 6, D).

To evaluate whether the potential mediators of phenotype development identified in our mouse model might also play a role in human subjects, we performed real-time PCR analysis in whole-blood samples of 4-year-old children of the LINA cohort. Interestingly, high urinary maternal MnBP concentrations (ie, MnBP concentrations greater than the median) were associated with a significant decrease in *ZFPML* expression in the children (MWU: $P = .025$; see Fig E3, A, in this article's Online Repository at www.jacionline.org) in accordance with our observations in the mouse model, no significant decrease of *FADS1* or *FANCA* expression in prenatally MnBP-exposed children was observed (data not shown). Concomitant with a decrease in *ZFPML* expression, asthmatic children showed a significant increase in methylation at a CpG site (chromosome 16: 88564360, $R = -0.87$, $P = .001$) located in an intronic human enhancer region corresponding to the region observed in mice, which also in human subjects appears to regulate its host gene *ZFPML* (see Fig E3, B and C).

Altered function of CD4⁺ T cells in the offspring of BBP-exposed mice

Both *Fanca* and *Zfpml* are described to play a crucial role in T-cell differentiation and function.^{36,37} To investigate a possible effect of maternal BBP exposure on both of these end points in the next generation, we isolated splenic CD4⁺ effector T cells from 8-week-old offspring and stimulated the cells with anti-CD3/CD28 antibodies. Although proliferation of T cells was not affected (data not shown), CD4⁺ T cells from BBP-exposed offspring appeared more activatable than T cells from nonexposed control mice, as shown by expression of the activation marker CD25 (Fig 7, A). Interestingly, we detected significantly more IL-4-producing CD4⁺ T cells in the F1 generation from exposed mice, whereas the increased number of IL-13-producing cells reached borderline significance and the number of IFN- γ -producing cells was unaffected (Fig 7, B). Furthermore, we found a reduced number of forkhead box p3 (Foxp3)-positive regulatory T cells in stimulated CD4⁺CD25⁺ T cells from BBP-exposed offspring compared with cells derived from control mice (Fig 7, C). However, the described differences did not reach statistical significance. Furthermore, the frequency of Foxp3-positive regulatory T cells in the blood and in unstimulated splenocytes of BBP-exposed offspring was not affected (see Fig E4 in this article's Online Repository at www.jacionline.org). Accordingly, the efficiency of CD4⁺CD25⁺ regulatory T cells to suppress responder T cells was slightly but not significantly impaired by using cells from BBP-exposed mice compared with regulatory T cells from control animals (Fig 7, D).

Maternal BBP exposure affects airway inflammation in the F2 generation

To investigate whether the asthma-promoting effect of perinatal BBP exposure persists up to the F2 generation, we mated perinatally exposed female with nonexposed male mice and induced an asthma-like phenotype in the F2 generation (Fig 2, C). Surprisingly, exposure to BBP during pregnancy and breastfeeding enhanced the eosinophilic airway inflammation (Fig 8, A and B) and AHR (Fig 8, C) but did not alter OVA-specific IgE levels in the F2 generation (Fig 8, D). Because treatment of F2 pups with Aza reduced their increased airway inflammation in relation to BBP exposure of their grandmother, an involvement

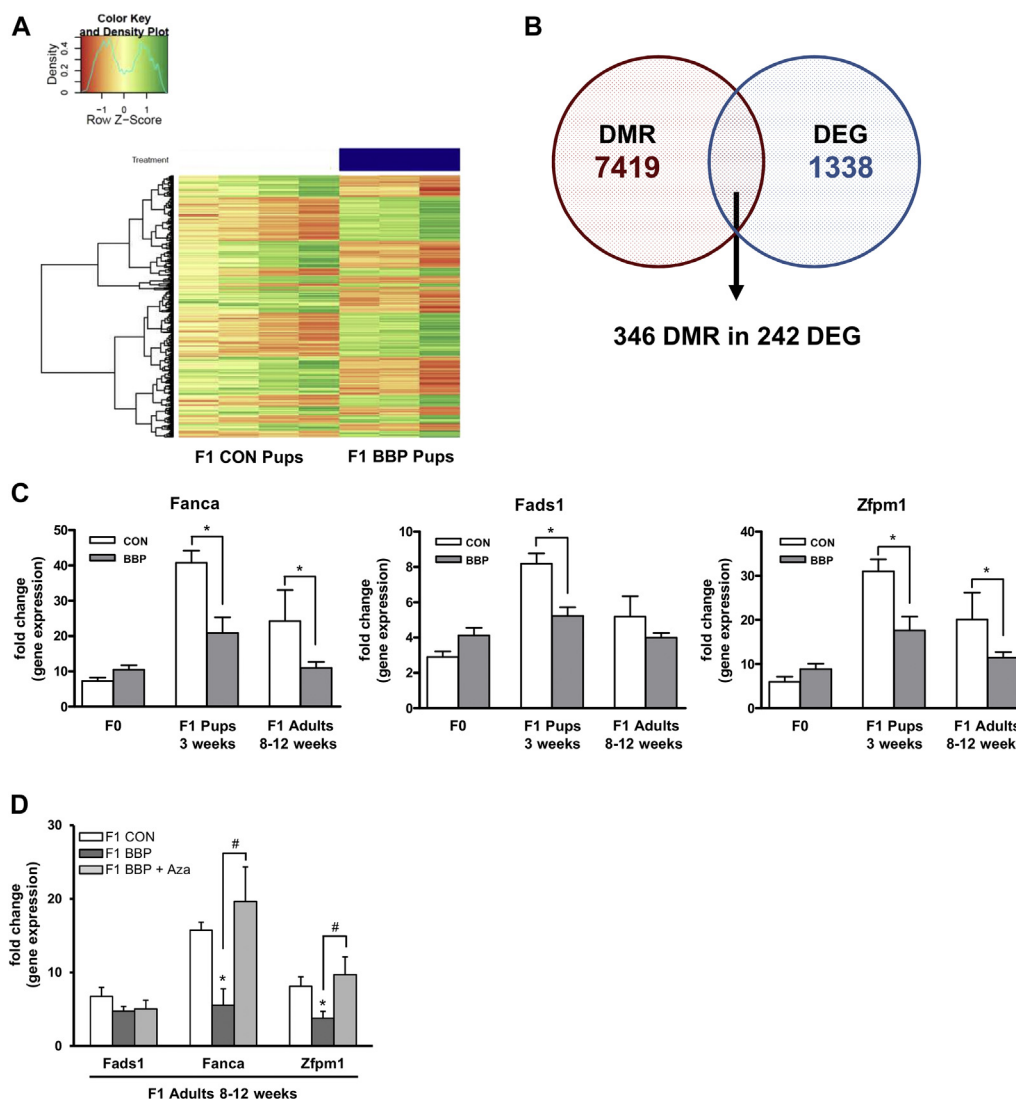


FIG 6. BBP-induced hypermethylation leads to downregulation of selected target genes. **A**, Heat map displays DEGs from pups of BBP-exposed dams versus control mice. **B**, Two hundred forty-two of 1338 DEGs in the offspring from BBP-exposed dams can be linked to 346 DMRs. **C**, RNA expression of selected genes in dams (n = 5), pups, or adult mice (n ≥ 6 from ≥4 dams) was verified by using quantitative PCR. **D**, Mice from BBP-exposed dams were treated with Aza, as described in the Methods section, and RNA expression was measured in mature offspring (n ≥ 6 from ≥2 dams). Data are expressed as means ± SEMs from at least 2 independent experiments. *P < .05, CON versus BBP. #P < .05, BBP versus BBP + Aza.

of epigenetic mechanisms, including DNA hypermethylation, in promoting this immune response is highly probable (Fig 8, A and C). However, we did not observe changes in DNA methylation related to BBP exposure in F2 pups in *Fads1*, *Fanca* (both promoters), or *Zfp1* DMRs (enhancer) identified in F1 pups, and these genes were not significantly differentially expressed (see Fig E5 in this article's Online Repository at www.jacionline.org).

DISCUSSION

The role of phthalate exposure in the increasing prevalence of atopic diseases has been a topic of great interest in recent years. However, from current studies, no clear conclusion can be drawn about the role of phthalates in asthma development or about the potential mechanisms involved.¹⁸ In the present study we showed that higher concentrations of MnBP in maternal urine during

pregnancy were associated with an increased risk of asthma in children up to age 6, whereas none of the other phthalate metabolites analyzed were associated with this disease outcome. These results are in line with findings of a recent study by Whyatt et al,⁴⁰ who found a positive association between the maternal urine phthalate metabolites MnBP and MBzP and asthma risk in children between the ages of 5 and 11,⁴⁰ whereas other studies only found such a correlation for MBzP but not MnBP.⁴¹ These discrepancies might in part be due to differences in study size but might also be related to regional differences in exposure pattern to the different members of the phthalate family. Interestingly, beyond the increased risk of asthma development by MnBP, we also observed a link between maternal MnBP concentrations and the risk for sensitization against inhalant allergens in the children, suggesting involvement of an atopic mechanism.

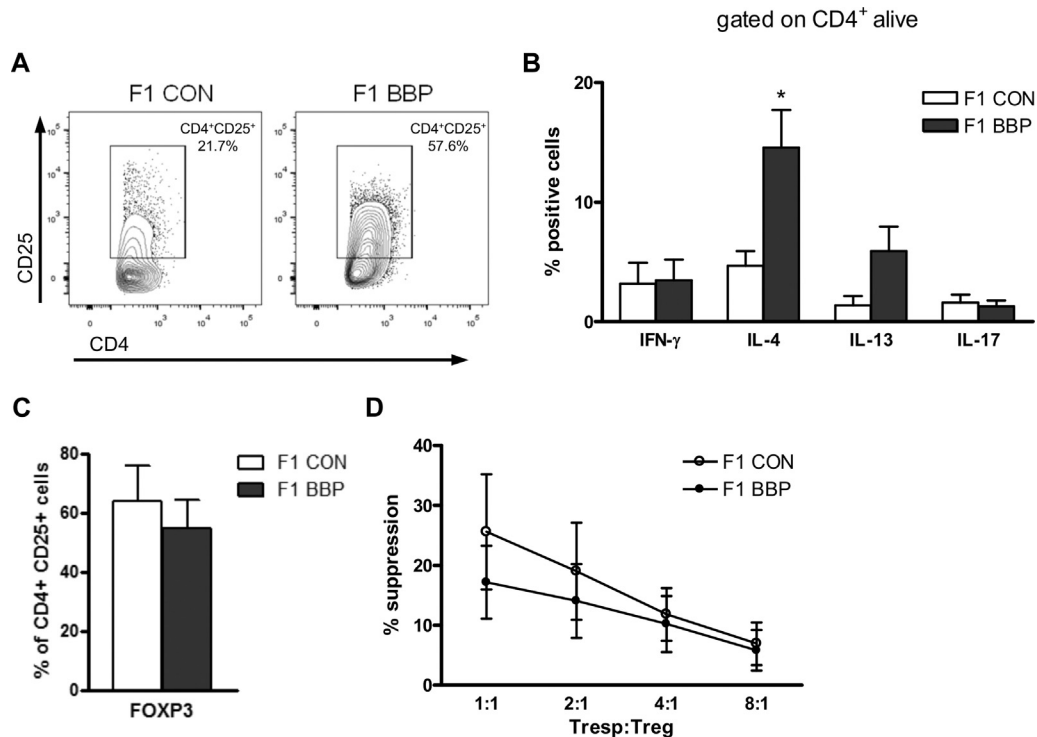


FIG 7. Altered function of CD4⁺ T cells in offspring of BBP-exposed mice. **A-C**, Numbers of CD4⁺CD25⁺ T cells (Fig 7, A), percentages of cytokine-producing T cells (Fig 7, B), and Foxp3⁺CD4⁺CD25⁺ T cells (Fig 7, C) are shown. **D**, Efficiency of CD4⁺CD25⁺ regulatory T (*Treg*) cells to suppress proliferation of responder T cells. Data are expressed as means \pm SEMs ($n \geq 5$ from 3 independent experiments). * $P < .05$.

MBzP and MnBP are both described as breakdown products of BBP,⁴⁰ with MnBP being the major metabolite of BBP and appearing in larger quantities in urine compared with MBzP.^{31,42} Thus we investigated the effect of maternal BBP exposure on the asthma-like phenotype in our murine transgenerational model.

Applied BBP exposure concentrations were based on the tolerable daily intake of BBP in human subjects of 0.5 mg/kg body weight per day,⁴³ which corresponds to an estimated uptake of 0.48 to 0.6 mg BBP/kg body weight per day in our mouse experiments (assuming 4–5 mL/d drinking water intake containing 3 μ g/mL BBP). The resulting urinary MnBP concentrations in our murine model were similar to those observed in highly exposed mothers of the LINA cohort, suggesting that BBP concentrations investigated here are representative for the real exposure situation in human subjects.

Although prenatal and perinatal exposure to BBP increased asthma severity in the offspring, exposure of adult mice did not affect the allergic immune response, further corroborating the notion that the most vulnerable time period for phthalate exposure is during pregnancy and shortly after birth.¹⁸

Another interesting finding was the inability of the BBP metabolites MnBP or MBzP to significantly increase allergic airway inflammation in the offspring when applied perinatally to the dams. These data point to a direct effect of the parent compound BBP, even without biotransformation. However, further insight into the BBP-mediated initial molecular events in the dams or the fetus has to be explored in future studies.

There is growing evidence that prenatal and perinatal exposure to diverse environmental chemicals might dysregulate the fetal or neonatal epigenome, with potential consequences for diseases manifesting in childhood.⁴⁴ Because the asthma-promoting effects by BBP in the present study were only detectable in the offspring from exposed dams, we investigated the effects of BBP on the epigenetic landscape. Previous studies already showed an effect on DNA methylation by certain phthalates but only based on targeted approaches restricted to preselected genes, such as glucose transporter type 4 (*Glut4*) or proinflammatory cytokines (*TNF- α*).^{45–47} In the current study we subjected samples from our transgenerational mouse model to a genome-wide evaluation of DNA methylation changes related to BBP. This analysis was centered on the CD4⁺ T-cell subset because these cells have a central role in initiation and maintenance of the allergic immune response.⁴⁸ Maternal BBP exposure led to global dysregulation in DNA methylation in the offspring dominated by DNA hypermethylation. In contrast to our findings, Wang et al⁴⁵ described a phthalate-induced DNA hypomethylation to be responsible for their observed increased asthma risk. However, the study was based on methylation analysis of 21 selected genes measured in human blood samples compared with our genome-wide approach in a T-cell subset. Nevertheless, we also observed phthalate-induced hypomethylation, such as in the promoter of IL-4 (chromosome 11: 53620267–53620300) similar to what has been described by Wang et al⁴⁵ in human subjects (data set 1). Counteracting the global DNA hypermethylation in our study by treating pups with the DNA methyltransferase inhibitor Aza prevented BBP-related exacerbation of allergic airway

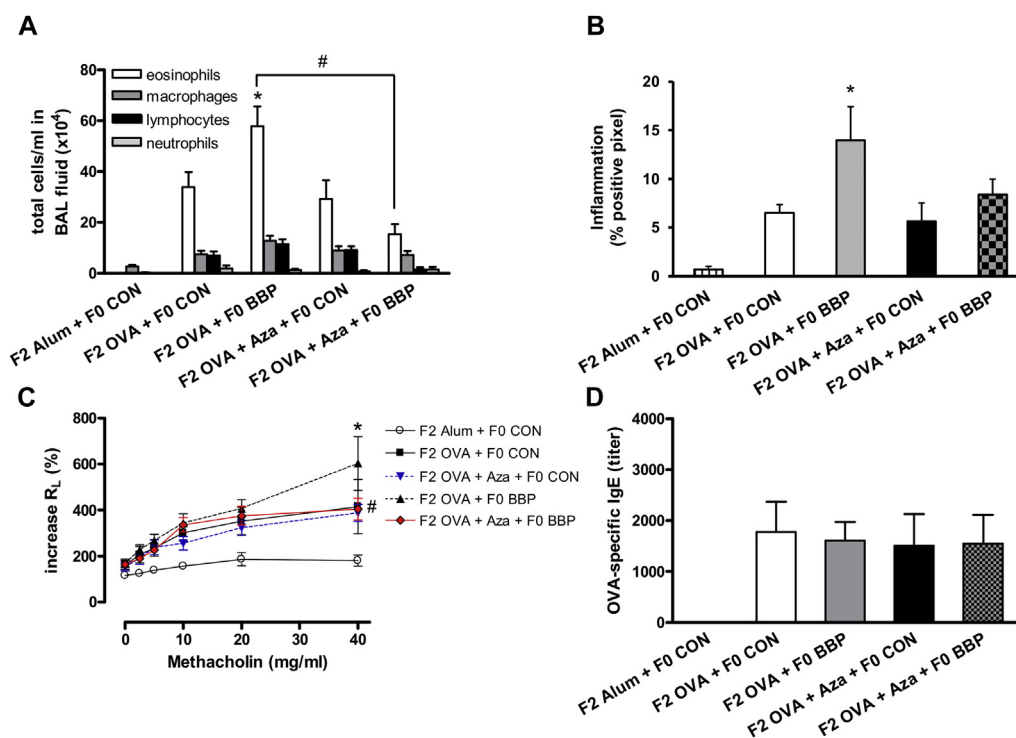


FIG 8. Maternal BBP exposure affects airway inflammation in the F2 generation. Total cell numbers in bronchoalveolar lavage (BAL) fluid (A), airway inflammation (B), lung resistance (C), and OVA-specific IgE levels (D) were examined in OVA-immunized F2 generation from prenatally BBP-exposed F1 dams compared with control mice. Data are expressed as means \pm SEMs ($n \geq 9$ animals per group in the F2 offspring [from ≥ 6 dams] from 6 independent experiments). * $P < .05$, OVA versus OVA + BBP. # $P < .05$, OVA + BBP versus OVA + BBP + Aza.

inflammation, suggesting a direct link between DNA hypermethylation and phenotype development.

Although recent findings indicated a wide variety of relationships between DNA methylation and gene expression, high promotor/enhancer methylation is classically associated with low levels of gene expression.^{49,50} Therefore we focused on significantly repressed genes with a promotor or enhancer hypermethylation to further elucidate the functional translation of the BBP associated hypermethylation. In the offspring of BBP-exposed dams, we identified 13 genes fulfilling these criteria, including 3 interesting candidates involved in immune processes contributing to T_H2 differentiation and therefore resulting in a higher susceptibility to experience allergic diseases.^{35,36,38} Considering the longitudinal stability of differential RNA expression induced by maternal BBP exposure from 3-week-old pups to the adult offspring, *Fanca* and *Zfpml* appeared to be the most promising genes. Characterizing the CD4⁺ T-cell function from offspring of BBP-exposed dams primarily revealed an increased susceptibility for a T_H2-driven immune response, as expected from downregulation of the GATA-3 repressor *Zfpml*.³⁷ In contrast, we could not show significant perturbations in regulatory T-cell function, which would have been one possible consequence of suppressed *Fanca* expression. Most interestingly, we could corroborate a possible mediating role of *Zfpml* in the phthalate effect by transferring the results of the mouse model to our LINA cohort. *ZFPML* mRNA expression was similarly decreased in children prenatally

exposed to high levels of MnBP. This decrease in gene expression was correlated with an increase in *ZFPML* methylation in the same region identified as a differentially methylated enhancer in our mouse model. The importance of reduced expression of the GATA-3 repressor *Zfpml* by maternal BBP exposure is supported by various studies therapeutically targeting GATA-3 to attenuate the T_H2-regulated inflammatory response.^{51,52}

To investigate whether the BBP-induced asthma-promoting effect functions also in the absence of direct environmental exposures through germline transmission of an altered epigenome,⁵³ we characterized asthma severity in the grandmotherly BBP-exposed F2 generation. Although we observed increased airway inflammation, the phenotype compared with the F1 generation was slightly different, showing no effect on IgE serum levels. Furthermore, neither DNA methylation nor RNA expression of *Fads1*, *Fanca*, and *Zfpml* were affected in the F2 generation, suggesting that the underlying mechanisms of an exacerbated asthma-like phenotype in the F1 and F2 generations are different.

In summary, data from our study strongly suggest that early exposure to specific phthalates increases susceptibility to the development of allergic asthma in the offspring and even contributes to airway inflammation in the F2 generation. The asthma-promoting effect was mediated by epigenetic changes, leading to altered expression of genes that play a crucial role in immune regulation. Therefore chemical exposure of the developing immune system leads to an altered epigenetic prenatal

programming, contributing not only to asthma development in later life but potentially also to other immune-regulated diseases.

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Key messages

- Maternal MnBP levels associate with an increased asthma risk in children.
- In mice maternal exposure to BBP enhances airway inflammation in the F1 and F2 generations through epigenetic alterations.

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